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14. ABSTRACT

The importance of p53 on prostate cancer is underlined by clinical observations that p53 alteration can be seen in most metastatic prostate cancers. The finding that re-introduction of wild-type p53 can cause growth arrest of prostate cancer cells further support the role of p53 in prostate tumor suppression. Therefore, to study on prostate tumor development and a therapeutic strategy targeting p53, it is necessary to understand how p53 is activated. To study this, we focus the p53 acetylation, which has been found as a potential mechanism of p53 activation, and investigate how acetylation controls the activity of p53. In this report, we provide the evidence that acetylation regulates p53 subcellular localization. Our study identifies acetylation as a novel mechanism that regulates p53 nucleus-cytoplasm trafficking by neutralizing C-terminal lysine residues, which in turn, controls the oligomerizationdependent nuclear export machinery. Although further investigations are needed to address the significance of acetylation-induced p53 trafficking in prostate cancer, our findings provide the basis for a more powerful therapy for prostate tumor suppression.

15. SUBJECT TERMS
p53, Tumor Suppressor gene, acetylation

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Introduction

Impairment of the activity of tumor suppressor p53 plays a critical role in most prostate cancers. In therapy, one critical problem is that most prostate cancers eventually become resistance to the standard androgen ablation hormonal therapy. Interestingly, it has been shown that androgen ablation-induced apoptosis in prostate gland is greatly diminished in the p53-deficient mouse (1). The role of p53 in prostate tumor development is further supported by finding that re-introduction of wild type p53 can cause growth arrest of prostate cancers cell lines (2). These results provide not only a potential molecular mechanism of resistance to hormone ablation therapy in advanced prostate tumors, which often harbor p53 mutations (3), but also a strategy of therapeutic agents that can enhance p53 activity for prostate tumor suppression. To study this, it is necessary to understand how p53 is activated. Recent finding show that p53 acetylation might play a role in p53 activation (reviewed in (4) (5)). Importantly, the signal transduction pathway leading to p53 actylation is functional in prostate cells, suggesting that this modification could be important for prostate tumor suppression. In this research, we will address to decipher the functional significance of p53 acetylation (**Aim1**). It has been shown that subcellular localization of p53 is important for p53 activation. Therefore, we will also address to characterize the role and significance of acetylation in regulating p53 subcellular localization (**Aim2**). Our research will provide important information on drag discovery targeting p53 activity by manipulating its acetylation state for prostate tumor suppression.

Body

To characterize the role and significance of acetylation in regulating p53 subcellular localization (Aim 2).

Although the importance of p53 intracellular trafficking in the regulation of p53 activity has been accumulating (6) (7) (8), little is known about how p53 intracellular trafficking is regulated. Surprisingly, we found that p300-mediated acetylation promotes cytoplasmic accumulation of p53. So far, we reported following evidence: 1) C-terminal lysine residues are required for acetylation-induced cytoplasmic accumulation of p53; 2) neutralization of positively charged lysine residues in C-terminus regulates subcellular localization of p53; 3) C-terminal lysine charge determines the oligomerization status of p53 (annual report 2004). Here we report new data supporting our idea that lysine acetylation can control p53 subcellular trafficking by regulating p53 oligomerization.

Overexpression of p300 does not affect the subcellular localization of a known nuclear peotein, HDAC1

So far, we have already demonstrated that overexpression of p300 induces p53 nuclear exit. To further verify whether overexpression of p300 specifically affects the localization of p53 and does not merely induce an overall re-localization of many proteins because of non-specific effects, we examined the subcellular localization of HDAC1, a nuclear protein, in p300 expressed cells. As shown in Figure 1, the subcellular localization of another nuclear protein, HDAC1, is not affected by the over-expression of p300 by either immuno-localization study (Figure 1A) or biochemical fractionation study (Figure 1B). This result suggested that ectopically expressed p300 specifically affects the localization of p53.

p300-mediated cytoplasmic accumulation of p53 requires the C-terminal NES

We have already demonstrated that p300-mediated p53 acetylation promotes p53 nuclear exit. As p53 actively shuttles between the nucleus and cytoplasm via a mechanism that involves a nuclear export signal (NES), we investigated whether acetylation-dependent p53 nuclear exit is dependent on the activation of the nuclear export machinery. As shown in Figure 2A(d) and B, the nuclear export inhibitor leptomycin B (LMB) efficiently blocked the cytoplasmic accumulation of p53 induced by p300. We also evaluated the subcellular localization of a p53 mutant whose C-terminal NES is inactivated. As shown in Figure 2A(e) and B, when expressed alone, NES mutant p53 is localized to the nucleus, similar to wild type p53. However, unlike wild type p53, NES mutant did not accumulate in the cytoplasm in respond to p300 expression and remained in the nucleus (Figure 2A(f) and B). Importantly, wild type and NES mutant p53 are acetylated to comparable levels (Figure 3). These results demonstrate that the C-terminal NES of p53 plays a dominant role in p300-mediated p53 nuclear exit. We have already shown that acetylation of C-terminal lysine residues inhibits p53 oligomerization. Since the accessibility of the C-terminal NES is regulated by the oligomerization status of p53, this new evidence further supports our idea that acetylation of C-terminal lysine residues regulates p53 oligomerization, which in turn, increases the accessibility of the p53 C-terminal NES.

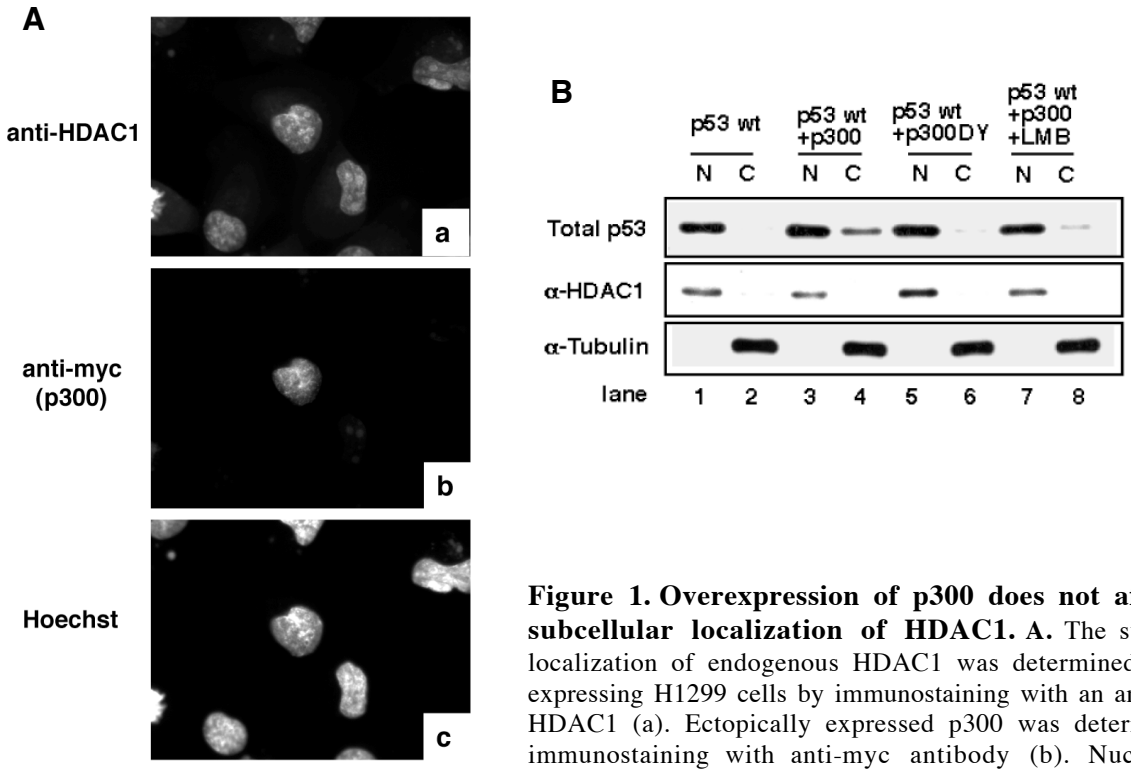
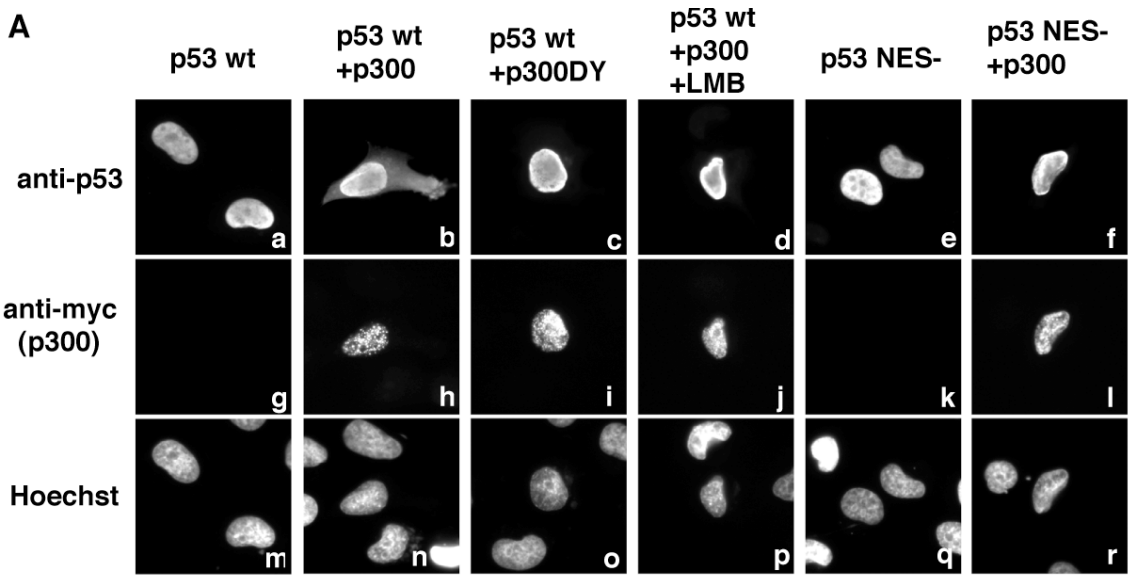


Figure 1. Overexpression of p300 does not affect the subcellular localization of HDAC1. A. The subcellular localization of endogenous HDAC1 was determined in p300 expressing H1299 cells by immunostaining with an antibody to HDAC1 (a). Ectopically expressed p300 was determined by immunostaining with anti-myc antibody (b). Nucleus was visualized by Hoechst 33258 (c). **B.** Levels of total p53, HDAC1 and tubulin in the nuclear (N) and cytoplasmic (C) fractions were determined by immunoblotting with antibodies to p53, HDAC1, and tubulin, respectively. Note that a known nuclear protein HDAC1 does not accumulate in cytoplasm in p300 expression cells (A(b) and B, lane 4).



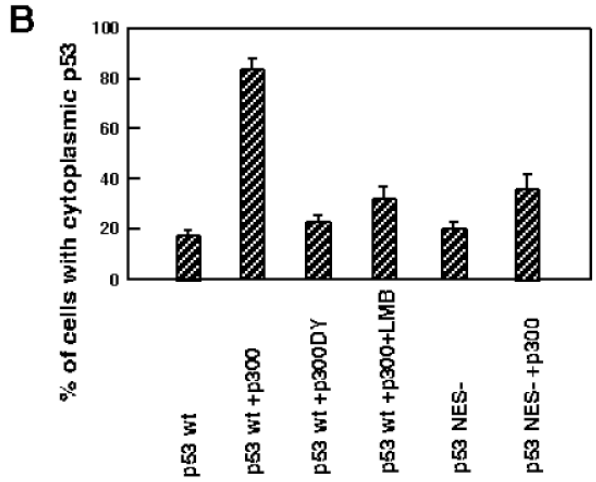


Figure 2. p300-mediated cytoplasmic accumulation of p53 requires the C-terminal NES. (A) Subcellular localization of wild-type and NES mutant p53 following transfection into p53 (-/-) H1299 cells. Cells were transfected with p53wt alone (a, g, m), p53wt and myc-p300 (b, d, h, j, n, p), p53wt and myc-p300DY (c, i, o), p53 NES(-) mutant alone (e, k, q), or p53 NES(-) mutant and myc-p300 (f, l, r) as indicated. The localization of p53, p300, and p300DY was determined by immunostainig with anti-p53 antibody and anti-myc antibody, respectively. 10 ng/ml of LMB was added 8h prior to immunostaining as indicated (d, j, p). Nucleus was visualized by Hoechst 33258 (m-r). (B) 100-200 cells from each transfection were scored. Results are an average of three independent experiments. Note that LMB, an inhibitor of nuclear export, and mutation of NES eliminated cytoplasmic accumulation of p53.

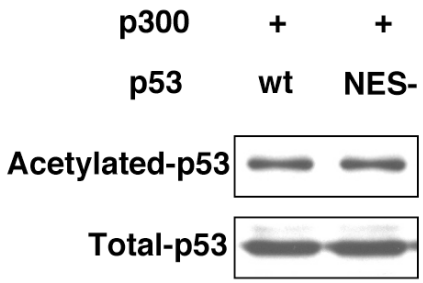


Figure 3. NES mutant p53 are acetylated to comparable level as wild-type. Levels of total p53, HDAC1 and tubulin in the nuclear (N) and cytoplasmic (C) fractions were determined by immunoprecipitation with anti-p53 antibody, followed by immunoblotting with anti-acetylated p53 antibody. Note that NES mutant also can be acetylated by p300 as same level as wild-type.

The biological significance of acetylation-mediated p53 nuclear exit

If p53 acetylation promotes its nuclear exit, what is the biological significance of acetylation-mediated p53 nuclear trafficking? Interestingly, recent evidence indicates that p53 can also promote apoptosis via a transcriptionally independent mechanism by directly interacting with apoptosis machinery in mitochondria (8). This surprising finding suggests the existence of a mechanism that would transport active p53 to the cytoplasm. One interesting possibility is that acetylation-mediated nuclear export might be involved in this process. To address this, we have been trying to determine if cytoplasmic p53 induced by acetylation can induce an association with mitochondria by co-immunostainig and biochemical fractionation. Also, we have been asking whether cytoplasmically localized acetylated p53 is essential for p53-induced apoptosis by examining the apoptotic activity of 6KR and 5KA mutants. This series of experiments would provide important insight into the function of acetylated p53 and the basis for a more powerful therapy for prostate tumor suppression.

Key Research Accomplishments

p300-mediated p53 acetylation promotes p53 nuclear exit specifically.

P300-induced p53 nuclear exit requires the C-terminal NES.

Reportable Outcomes

Kawaguchi, Y., et. al., Charge modification at multiple C-terminal lysine residues regulates p53 oligomerization and its nucleus-cytoplasm trafficking, J. Biol. Chem. (in press).

Conclusions

The understanding of p53 activation provides important information on the strategy for treatment for prostate cancer. We present the evidence further support an idea that acetylation promotes p53 nuclear exit that might be contribute to activate apoptosis machinery in mitochondria. Although the importance of acetylation-mediated p53 nuclear export in prostate tumor suppression still awaits further investigation, our study identifies acetylation as a potential novel mechanism that regulates p53 activity by control p53 intracellular trafficking.

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Appendices

CHARGE MODIFICATION AT MULTIPLE C-TERMINAL LYSINE RESIDUES REGULATES p53 OLIGOMERIZATION AND ITS NUCLEUS-CYTOPLASM TRAFFICKING

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Running title: Acetylation regulates p53 trafficking

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The basal level of the tumor suppressor p53 is regulated by MDM2-mediated ubiquitination at specific lysines, which leads to p53 nuclear export and degradation. Upon p53 activation, however, these lysines become acetylated by p300/CBP. Here we report an unexpected finding that p300-mediated acetylation also regulates p53 subcellular localization and can promote cytoplasmic localization of p53. This activity is independent of MDM2 but requires a p53 nuclear-export signal (NES) and acetylation of multiple lysines by p300. Mechanistically, we show that conversion of a minimal four of these lysines to alanines but not arginines mimics p300-mediated p53 nuclear export, and these lysine-neutralizing mutations effectively prevent p53 tetramerization, thus exposing the oligomerization-regulated NES. Our study suggests a threshold mechanism whereby the degree of acetylation regulates p53 nucleus-cytoplasm trafficking by neutralizing a lysine-dependent charge patch, which in turn, controls oligomerization-dependent p53 nuclear export.

The tumor suppressor p53 plays critical roles in protecting cells from malignant transformation. Upon its activation in response to a variety of stress stimuli, p53 becomes stabilized and accumulates in the nucleus and induces cell cycle arrest and apoptosis (1). Due to its potent growth inhibition and pro-apoptotic activity, p53 is normally kept latent and at low levels in unstressed cells by its negative regulator MDM2. MDM2 represses p53, in part, by promoting p53 degradation and nuclear export through catalyzing p53 poly- and mono- ubiquitination on specific lysine residues (2). Although poly-ubiquitinated p53 is targeted to proteasome for degradation, the exact mechanism by which mono-ubiquitination promotes p53 nuclear export is not understood.

In contrast to MDM2 mediated-ubiquitination, acetylation has been positively linked to both p53 transcriptional activity and stability (reviewed in (3,4)). Consistent with this idea, p53 invariably becomes acetylated upon its activation by various p53-activating agents (5). The acetylation of p53, which is catalyzed by the p300/CBP acetyltransferases, occurs on at least six lysine residues clustered at the C-terminus (6,7). It is unclear why so many lysines are modified by acetylation. Nor is it known whether the acetylation of the lysines provides a functional moiety for protein-protein interaction or serves to modify the conformation of p53. One clue as to the function of p53 acetylation lies in the potentially interesting links between acetylation and ubiquitination. Both the acetylation and ubiquitination machinery modify the ε amino group of the lysine residue. Furthermore, acetylation and ubiquitination occur on a common set of lysine residues at the C-terminus of p53 (7). These observations suggest that MDM2-mediated ubiquitination and p300/CBP-mediated acetylation might functionally antagonize one another to control p53 activity.

Recent studies have highlighted the importance of intracellular trafficking in the regulation of p53 activity (8-10). It is known that p53 actively shuttles between the nucleus and cytoplasm via a mechanism that involves a nuclear export signal (NES) and its receptor Crm-1 (reviewed in (11)). Although its importance is apparent, little is known about how p53 nuclear export is regulated. Recent studies have indicated that the oligomeric status of p53 may control the accessibility of NES to the export machinery (8). Biochemical and structural studies revealed that the C-terminal NES is normally exposed in the inactive monomeric or dimeric forms of p53 that are subject to active nuclear export. Upon its conversion to the active tetrameric form, however, the C-terminal NES becomes buried and inaccessible, resulting in p53 nuclear retention. At present, it is not understood how the oligomerization status of p53 is regulated in response to specific stress signals or modifications, such as ubiquitination.

Here we present evidence that lysine acetylation is a novel mechanism that controls p53 oligomerization and subcellular trafficking. We find that p53 hyperacetylation leads to cytoplasmic accumulation of endogenous p53 in response to p53-activating agents. We further show that over-expression of p300 stimulates cytoplasmic accumulation of p53 in an acetylation-dependent but MDM2-independent manner. Mechanistically, we provide evidence suggesting that acetylation promotes the nuclear export of p53 by neutralizing the "charge patch" created by the C-terminal lysine residues. The charge neutralization of the C-terminal lysines prevents p53 oligomerization and therefore exposes the p53 NES, allowing efficient p53 export. The requirement of multiple modified lysines for efficient export suggests a potential threshold mechanism wherein the acetylation level of p53 serves as a signal that promotes p53 export to the cytoplasm.

Materials and Methods

Cell lines and transfection - H1299 human cells, p53(-/-), MDM2(-/-) mouse embryonic fibroblast (MEF) cells, and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM). All cells were grown at 37 ^{0}C in the presence of 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO2. All transfections were performed by the calcium phosphate method as described previously (12).

Plasmids - Wild-type human p53 cDNA, wild–type human MDM2, wild-type human myc-p300 and human p300DY mutants were described previously (5). p53-5KR and -6KR mutants were generated using site-directed mutagenesis, changing lysines 320, 370, 372, 373, 381 and 382 to arginines. P53-2KA, -3KA, -4KA and –5KA mutants were also constructed using site-directed mutagenesis to exchange the lysines to alanines. p53-NES(-) mutants were also constructed using site-directed mutagenesis to change the leucines (a.a 348 and 350) to alanines.

Immunofluorescence - For immunofluorescence staining, cells grown on a glass coverslip were transfected with 0.1 μg p53 and 1μg myc-epitope tagged p300 expression plasmids. Immunostaining was performed as described (12) using anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz), anti-HDAC1 monoclonal antibody (H-11, Santa Cruz), anti-myc monoclonal antibody 9E10 and anti-MDM2 monoclonal antibody SMP-14 (Santa Cruz). Hoechst 33258 was used to visualize the nucleus.

Fractionation, Immunoprecipitation and immunoblotting - The cells were homogenized using a dounce homogenizer in buffer [25 mM Hepes-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA, 5mM MgCl2, 50 mM NaF, 1mM dithiothreitol (DTT)] supplemented with 5 mM of deacetylase inhibitor TSA (Sigma) and protease inhibitors. After centrifugation at 960g for 5 min, nuclei pellets were washed with the homogenizer buffer, and then lysed in buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DDT] supplemented with 5 mM TSA and protease inhibitors. Lysates were centrifuged at 5000g for 5 min to obtain supernatants of nuclear fraction. While, cytoplasmic supernatants were added NaCl and NP-40 to bring up to 150 mM and 1%, respectively. Immunoprecipitation and immunoblotting were performed as described previously (5). Proteins were detected with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys373) p53 antibody, anti-human acetylated (Lys382) p53 antibody (Calbiochem), anti-α-

tubulin antibody (DM1A, Sigma) or anti-green fluorescent protein (GFP) antibody (Boehringer Mannheim).

Protein production and oligomerization assay - Wild-type, 3KA, 4KA or 4KR p53 C-terminal DNA (amino acid 326-393) including the tetramerization domain was cloned into pGEX-6P-1 vectors. GST fusion constructs were expressed in *E.coli*, and then purified by Glutathione Sepharose 4B (Amersham) and cleaved with Precission protease (Amersham). The oligomerization assay was performed as described previously (8).

Results

Acetylation regulates subcellular localization of p53

We have previously shown that p53 becomes acetylated by p300/CBP upon cellular stresses (5). As p53 activation is accompanied by its accumulation in the nucleus, we asked whether p300-mediated acetylation regulates p53 subcellular localization. To test this, we transfected p53-null H1299 cells with expression plasmids for p53 and p300, and then examined p53 subcellular localization. As shown in Figure 1A(a), when expressed alone, p53 resides almost exclusively in the nucleus. To our surprise, upon co-expression with p300, p53 localized to both the nucleus and the cytoplasm in the majority of the cells expressing both transfected p53 and p300 (Figure 1A(b), and B). Cell counts demonstrated that up to 84% of the cells that expressed both p53 and p300, showed cytoplasmic accumulation of p53 (Figure 1B). To determine if the accumulation of p53 in the cytoplasm requires p300 activity, we examined whether an acetyltransferase-deficient p300DY mutant (5) could affect the subcellular localization of p53. As shown in Figure 1A(c) and B, unlike wild type p300, the acetyltransferase-deficient p300DY mutant did not stimulate p53 accumulation in the cytoplasm. As expected, both p300 and p300DY are localized to the nucleus (Figure 1A(h, i, j, l), and neither p300 nor the p300DY mutant had an effect on the levels of p53 under the experimental conditions (Figure 1D, and (5)). Together, these results indicate that p300-mediated cytoplasmic accumulation of p53 requires p300-acetyltransferase activity.

As p53 subcellular localization is regulated by active nuclear export, we investigated whether acetylation-dependent p53 accumulation in the cytoplasm is dependent on the activation of the nuclear export machinery. As shown in Figure 1A(d) and B, the nuclear export inhibitor leptomycin B (LMB) efficiently blocked the cytoplasmic accumulation of p53 induced by p300. We also evaluated the subcellular localization of a p53 mutant whose C-terminal nuclear export signal (NES, (8)) is inactivated. As shown in Figure 1A(e) and B, when expressed alone, NES mutant p53 is localized to the nucleus, similar to wild type p53. However, unlike wild type p53, NES mutant did not accumulate in the cytoplasm in respond to p300 expression and remained in the nucleus (Figure 1A(f) and B). Importantly, wild type and NES mutant p53 are acetylated to comparable levels. These results demonstrate that p300-mediated cytoplasmic accumulation of p53 requires the C-terminal NES and suggest that acetylation enhances p53 nuclear export.

To further verify the conclusions from the immuno-localization study, we determined the subcellular localization of p53 by biochemical fractionation. As shown in Figure 1C, p53 is normally a nuclear protein (Lane 1-2). However, co-expression of wild type p300, but not the p300DY mutant, induced a marked accumulation (25% of the total p53) of the p53 protein in the cytoplasmic fraction (compare Lanes 2, 4 and 6). The p300-dependent cytoplasmic accumulation of p53 could again be largely reversed by LMB treatment (Lane 8). These results are in agreement with the data obtained from the immuno-localization, providing further evidence that p300-mediated acetylation affects subcellular localization of p53. The presence of acetylated p53 in the cytoplasmic fraction suggests that p53 can be exported to the cytoplasm after being acetylated (Figure 1C).

Lastly, we asked if endogenous p53 accumulates in the cytoplasm during stress stimuli. As shown in Figure 1E, we observed significant accumulation of endogenous p53 in the cytoplasm in response to UV-irradiation or hypoxia when deacetylation of p53 is suppressed (compare Figure 1E(b, d) with 1E(a, c)). In agreement with immuno-localization analysis, subcellular fractionation assays also reveal significant accumulation of endogenous p53 in the cytoplasm

in response to UV-irradiation (Figure 1F, lane 3). Importantly, cytoplasmic p53 induced by UV-irradiation is acetylated, as it can be recognized by an antibody specific for the acetylated p53 (Figure 1F, lane 3, upper panel). Together, these results support the conclusion that hyperacetylation of p53 leads to its accumulation in the cytoplasm.

p300-mediated cytoplasmic accumulation of p53 is independent of MDM2

It was reported that MDM2 stimulates p53 nuclear export by promoting p53 ubiquitination (13,14). As p300 functionally interacts with MDM2(15), we determined whether p300-induced cytoplasmic accumulation of p53 requires MDM2. To test this idea, we expressed p53 alone or together with p300 in MDM2-deficient MEF cells (16), and assessed p53 subcellular localization. As shown in Figure 2A, in the absence of MDM2, p300 is still capable of stimulating the cytoplasmic accumulation of p53. In fact, more than 90% of cells over-expressing p300 showed p53 in cytoplasm, which is similar to the effect induced by MDM2 (Figure 2B). This result indicates that p300-mediated cytoplasmic accumulation of p53 is independent of MDM2.

C-terminal lysine residues are required for efficient acetylation-induced cytoplasmic accumulation of p53

The results presented so far support the idea that p300 affects subcellular localization of p53 in an acetylation-dependent manner, likely by directly acetylating p53. To further investigate this possibility, we determined whether lysine (K) residues known to be acetylated by p300 are required for acetylation-induced cytoplasmic accumulation of p53. We, therefore, mutated multiple lysines to arginines (5KR and 6KR, Figure 3A) that are known targets of acetylation by p300 (7) and evaluated the subcellular distribution of these mutants in response to p300. As shown in Figure 3B, when expressed alone, the localization of these p53 KR mutants is almost entirely nuclear and indistinguishable from wild type p53. However, in response to ectopically expressed p300, the number of cells that show a cytoplasmic accumulation of the 5KR (51%) and 6KR (30%) p53 mutants is markedly reduced compared to that of wild type p53 (84%). These data indicate that the lysine residues acetylated by p300 are required for p53 nuclear exit in response to p300.

Neutralization of positively charged lysine residues in the C-terminus regulates subcellular localization of p53

It was previously shown that acetylation in histone H2A.Z works by neutralizing the charge conferred by multiple lysine residues (17). As acetylation also targets multiple lysine residues in p53, we asked whether acetylation promotes p53 nuclear exit by a similar mechanism. To test this, we generated charge-neutralizing mutations by converting lysine (K) residues known to be acetylated and ubiquitinated to alanine (A), either individually or in combination, and evaluated their subcellular localization. As shown in Figure 4, the subcellular localization of the 2KA and several 3KA (3KA-1, 3KA-2 and 3KA-3) mutants is similar to that of wild type p53 and is mostly nuclear (Figure 4B and C). In contrast, when four lysine residues are mutated in three different combinations (4KA-1, 4KA-2 and 4KA-3), these p53 mutants clearly began to accumulate in the cytoplasm. The conversion of five lysine residues (5KA) led to a further increase in the number of cells with cytoplasmic p53 staining (Figure 4B and C). The cytoplasmic localization of the 4KA-1, 4KA-2, 4KA-3 and 5KA p53 mutants was observed in 51%, 52%, 48% and 64% of transfected cells, respectively, compared with 19% for wild type p53 (Figure 4C). Furthermore, the intensity of the wild type p53 protein detected in the cytoplasm was much weaker than that of the 4KA and 5KA mutants (data not shown). Importantly, cytoplasmic p53 4KA and 5KA mutants can be effectively eliminated upon treatment with LMB (Figure 4B and C), supporting the idea that nuclear export is required for cytoplasmic accumulation of the 4KA and 5KA p53 mutants. Together, these observations indicate that the degree of p53 cytoplasmic accumulation is proportional to the number of lysine residues neutralized and suggest that acetylation modulates p53 subcellular localization by modifying the positive charge of specific lysine residues at the C-terminus of p53.

C-terminal lysine charge determines the oligomerization status of p53

The accessibility of the C-terminal NES to the export machinery has been shown to be regulated by the oligomerization status of p53 (8). We therefore assessed whether modification of the lysine charge activates p53 export by regulating p53 oligomerization status. Based on the observation that the conversion of at least four but not three lysines to alanines promotes p53 cytoplasmic accumulation, recombinant wild type, 3KA, and 4KA (4KA 1-3, Figure 4A) mutant polypeptides encompassing the entire p53 tetramerization domain and lysine rich C-terminus (amino acids 326-393) were tested for their ability to oligomerize. A mutant p53 with four lysines converted to arginines (4KR), which would prevent acetylation but preserve the charge of the lysine, was used as an additional control. As shown in Figure 5, the wild type and 3KA polypeptides dimerized and tetramerized readily (Lanes 2 and 4). In contrast, the 4KA-1, 4KA-2 and 4KA-3 polypeptides completely failed to do so (Lane 6, 8 and 10). Importantly, polypeptides from the 4KR mutant, which is a nuclear protein (data not shown), showed a wild type capacity to oligomerize (Lane 12). Together, these results demonstrate that the oligomerization status of p53 can be controlled by the charge conferred by a defined number of lysine residues.

Discussion

In this report, we provide evidence that acetylation regulates p53 subcellular localization, at least in part, by activating its nuclear export. Our study identifies the charge of C-terminal lysine residues targeted by p300 as a regulatory element that controls p53 oligomerization and subcellular localization. Our data suggest that acetylation regulates p53 nucleus-cytoplasm trafficking by neutralizing the lysine charge patch, which in turn controls oligomerization-dependent p53 nuclear export.

The subcellular localization of p53 is believed to be controlled at the levels of its oligomerization status. It was proposed that tetramerization prevents both p53 nuclear import and export (reviewed in (18)). In the case of nuclear export, as the dominant C-terminal NES is located in the oligomerization domain, it was suggested that the accessibility of the p53 NES is regulated by the oligomerization status (8). This conclusion is supported by the solution and the crystal structure of the oligomerization domain, which demonstrates that the C-terminal NES is exposed in monomeric or dimeric conformations but it is buried in p53 tetramers (19-21). The inter-conversion between the tetrameric and monomeric states, which corresponds to p53 transcriptional activity, would therefore determine the availability of the NES and, consequently, the efficiency of p53 export. However, the biochemical basis that controls p53 oligomerization and its regulation are poorly understood. In this report, we provide experimental evidence that the charge of lysine residues targeted by acetylation and ubiquitination machinery may be a key determinant of p53 oligomerization. This conclusion is supported by the observation that mutations that neutralize four acetylatable lysine residues result in p53 that cannot oligomerize (Figure 5). Further, these charge-neutralizing mutations also lead to p53 accumulation in the cytoplasm (Figure 4). Lastly, these cytoplasmic accumulations of p53 can be effectively reversed by the nuclear export inhibitor LMB (Figure 1 and 4). Our data, however, do not exclude the possibility that the charge neutralization of lysine residues might also affect p53 nuclear import as well. Regardless of which mechanism might play a more dominant role, our results support the idea that a charge modification of the lysine residues is a critical determinant in regulating p53 subcellular localization.

If C-terminal charge plays a critical role in p53 nuclear export, how is this charge patch regulated? Our study identifies acetylation as one potential mechanism that regulates the lysine charge patch. Acetylation occurs at the ε-amino group of a lysine. Thus acetylation would result in a loss of the positive charge, which in turn would affect p53 oligomerization (Figure 5) and nucleus-cytoplasm trafficking (Figure 4). The idea that acetylation regulates p53 export by neutralizing charge is supported by an elegant study on histone H2A.Z in which acetylation was shown to function by modifying a charge patch also made of multiple lysines (17). In this regard, ubiquitin also modifies the ε-amino group of a lysine. However, given that a fusion of mono-ubiquitin to p53 is sufficient to promote p53 nuclear export (22), acetylation and ubiquitination would likely regulate p53 export through different mechanisms.

Interestingly, although the conversion of different combinations of four lysines known to be targets of acetylation to alanines (4KA mutants) promotes cytoplasmic accumulation of p53, the conversion of only three lysines (3KA) has little effect (Figure 4). In support of this observation, we show that p53 4KA mutants fail to tetramerize, while the 3KA mutants do as efficiently as the wild type (Figure 5). These results suggest that there might be a threshold for the activation of p53 export, as determined by the overall charge provided by the lysine cluster at the C-terminus. Thus, the extent of charge neutralization by acetylation could in theory determine the subcellular distribution of the activated p53. We suspect that, upon stresses, p53 would be stabilized in the nucleus when less than three lysine residues are acetylated; however when more than four lysine residues are acetylated, presumably in response to prolonged or intense insults, p53 becomes hyperacetylated and exported to the cytoplasm.

What is the biological significance of acetylation-mediated p53 nuclear exit? All evidence so far indicates that acetylation positively regulates p53 function (4). Thus, it is logical to speculate that hyperacetylated p53 has a function when it is delivered to the cytoplasmic compartment. Interestingly, recent evidence indicates that p53 can also promote apoptosis by directly interacting with the apoptosis machinery in mitochondria(10). Although a recent study observed very similar profiles in the acetylation level on lysine 382 and 372 of nuclear and mitochondrial p53 and thus concluded that acetylation is not the determining factor for mitochondria targeting (23), it remains possible that the total acetylation of mitochondrial p53 might still be up-regulated via other acetylatable lysine residues. Alternatively, the hyperacetylated cytoplasmic p53 might undergo rapid deacetylation before they reach mitochondria. Although the importance of acetylation-mediated p53 nuclear export in tumor suppression still awaits further investigation, our study identifies acetylation as a potential mechanism that controls p53 intracellular trafficking in response to stresses.

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FOOTNOTES

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The abbreviations used are: NES, nuclear export signal; LMB, leptomycin B

FIGURE LEGENDS

Figure 1. *Acetylation regulates p53 nuclear export.* **(A)** Subcellular localization of p53 and p300 following transfection into p53 (-/-) H1299 cells. Cells were transfected with p53wt alone (a, g, m), p53wt and myc-p300 (b, d, h, j, n, p), p53wt and myc-p300DY (c, i, o), p53 NES(-) mutant alone (e, k, q), or p53 NES(-) mutant and myc-p300 (f, l, r) as indicated. The localization of p53, p300, and p300DY was determined as described in Materials and Methods. 10 ng/ml of LMB was added 8h prior to immunostaining as indicated (d, j, p). Nucleus was visualized by Hoechst 33258 (m-r). **(B)** Acetylation by p300 increases the percent of cells with cytoplasmic p53. 100-200 cells from each transfection were scored. Results are an average of three independent experiments. **(C)** Cytoplasmic p53 is acetylated. Levels of acetylated p53 in the nuclear (N) and cytoplasmic (C) fractions were determined by immunoprecipitation with anti-p53 polyclonal antibody, followed by immunoblotting with anti-acetylated p53 antibody. Levels of total p53, HDAC1, and α-tubulin were assayed by blotting with anti-p53 monoclonal antibody, anti-HDAC1 monoclonal antibody and anti-tubulin monoclonal antibody. **(D)** p300 does not change the expression level of total p53. GFP was used as internal control, and detected by anti-GFP monoclonal antibody. **(E)** A549 cells were irradiated to UV (75J/m2)(a,b), or treated with deferoxamine (DFX) to induce hypoxia(c,d). After UV irradiation or hypoxia treatment, the cells were treated with or without TSA and nicotinamide (Nico) for 8h, and then immunostained with anti-p53 antibody. **(F)** Cytoplasmic accumulation of endogenous p53 after UV exposure and p53 acetylation were determined as described in Figure 1C.

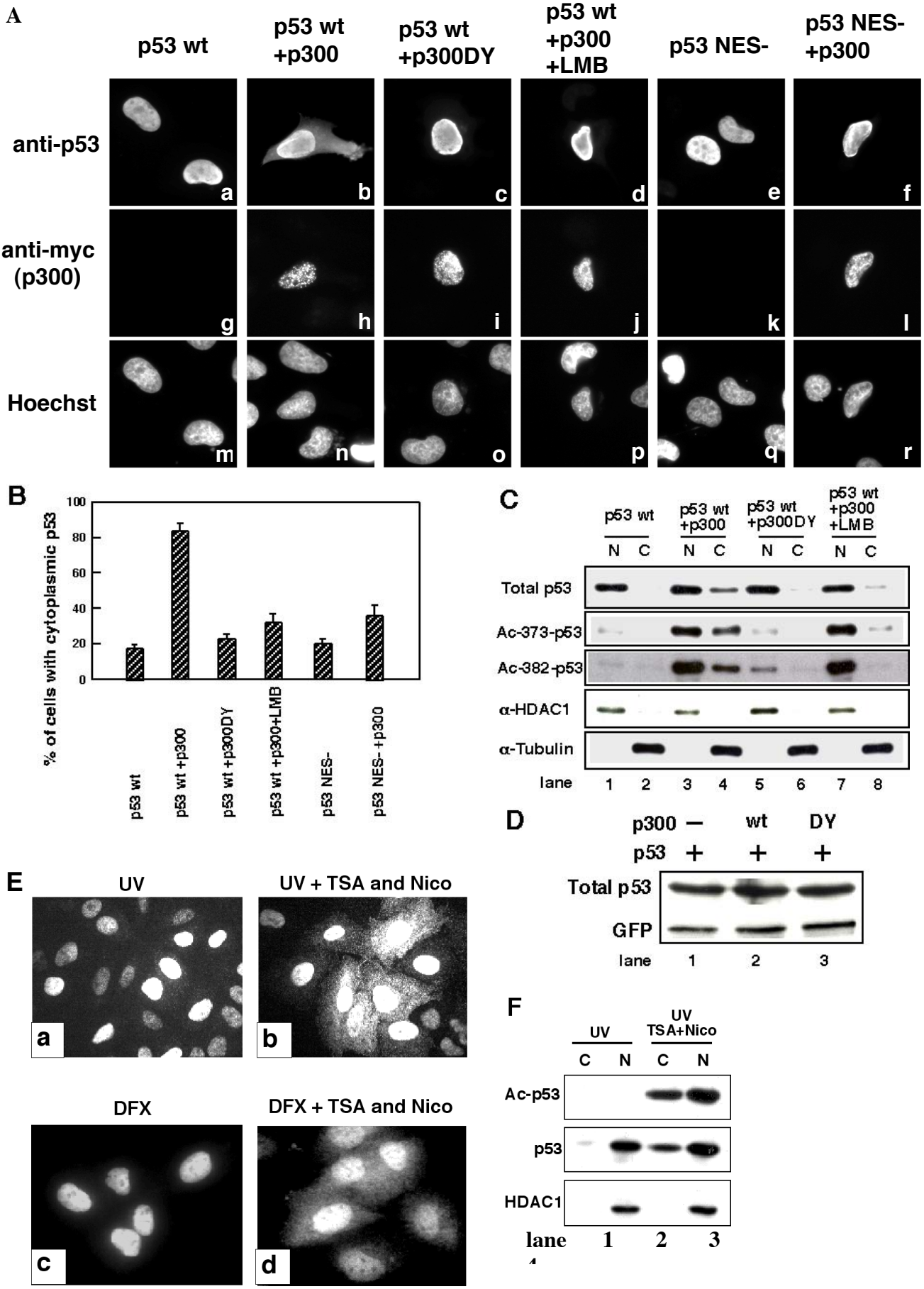
Figure 2. *Acetylation-mediated p53 nuclear export is not MDM2-dependent.* **(A)** Subcellular localization of p53, p300 and MDM2 following transfection into p53(-/-) / MDM2(-/-) MEF cells. p53 (a,b,c) and p300 (d) were detected as described in Figure 1A. MDM2 (e) was detected by immunostaining with the anti-MDM2 monoclonal antibody (SMP-14). **(B)** The percent of cells with cytoplasmic p53 was determined as described in Figure 1B.

Figure 3. *The C-terminal lysines of p53 are involved in the acetylation-mediated nuclear export of p53.* **(A)** Schematic representation of wild-type p53 and an expanded view of the C-terminus outlining the lysine (K) to arginine (R) mutations of p53, 5KR, and 6KR mutants. Abbreviations are as follows: TAD, transactivation domain; DNA-BD, DNA binding domain; OLIGO, oligomerization domain **(B)** The loss of available acetylation sites results in a reduction in the percentage of cells with cytoplasmic p53. Wild-type p53 or the KR mutants were transfected into H1299 cells alone or co-transfected with p300 as indicated. The percent of cells with cytoplasmic p53 was determined as in Figure 1B.

Figure 4. *Neutralization of positively charged lysine residues in the C-terminus of p53 regulates subcellular localization of p53.* **(A)** Schematic representation of the p53 lysine (K) to alanine (A) mutants. **(B)** Subcellular localization of wild-type p53 and KA mutants transfected into H1299 cells. LMB treatment was performed as described in Figure 1A. Nucleus was visualized by Hoechst 33258. **(C)** An increase in the number of KA mutations results in an increase in the percent of cells with cytoplasmic p53. Results were determined as in Figure 1B.

Figure 5. *Neutralization of positively charged lysine residues in the C-terminus determines the oligomerization status of p53.* Wild-type (lane 1, 2), 3KA (lane 3, 4), 4KA (lane 5, 6) or 4KR (lane 7, 8) mutant p53 polypeptides consisting of the tetramerization domain (amino acid 326-393) were expressed in *E.coli* as GST fusions. Ten micrograms of GST-cleaved protein were incubated at 37 ºC with or without 0.1% glutaraldehyde for 15 min, then analyzed by a 20% SDS-PAGE to separate the p53 monomer from the oligomers.

Figure1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p53 wt +p300
p53 wt +p300DY
p53 wt +p300 +LMB
p53 NES-
p53 NES- +p300
anti-p53
anti-myc (p300)
Hoechst
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
B
% of cells with cytoplasmic p53
100
80
60
40
20
0
p53 wt
p53 wt +p300
p53 wt +p300DY
p53 wt +p300+LMB
p53 NES-
p53 NES-+p300
C
p53 wt
p53 wt +p300
p53 wt +p300DY
p53 wt +p300 +LMB
N C N C N C N C
Total p53
Ac-373-p53
Ac-382-p53
α-HDAC1
α-Tubulin
lane 1 2 3 4 5 6 7 8
D
p300 – wt DY
p53 + + +
Total p53
GFP
lane 1 2 3
E
UV
UV + TSA and Nico
DFX
DFX + TSA and Nico
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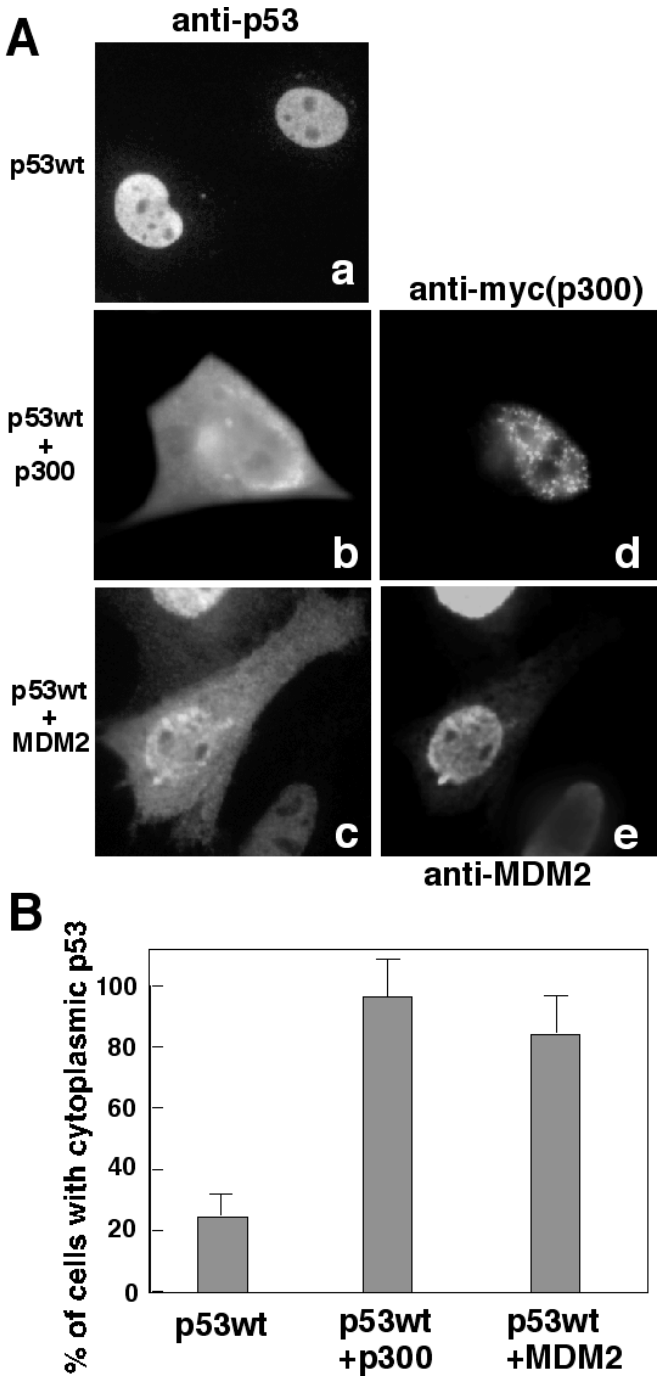
b
c
d
F
UV
UV TSA+Nico
C N C N
Ac-p53
p53
HDAC1
lane 1 2 3

Figure2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p53wt
a
anti-myc(p300)
p53wt
+
p300
b
d
p53wt
+
MDM2
c
e
anti-MDM2
B
% of cells with cytoplasmic p53
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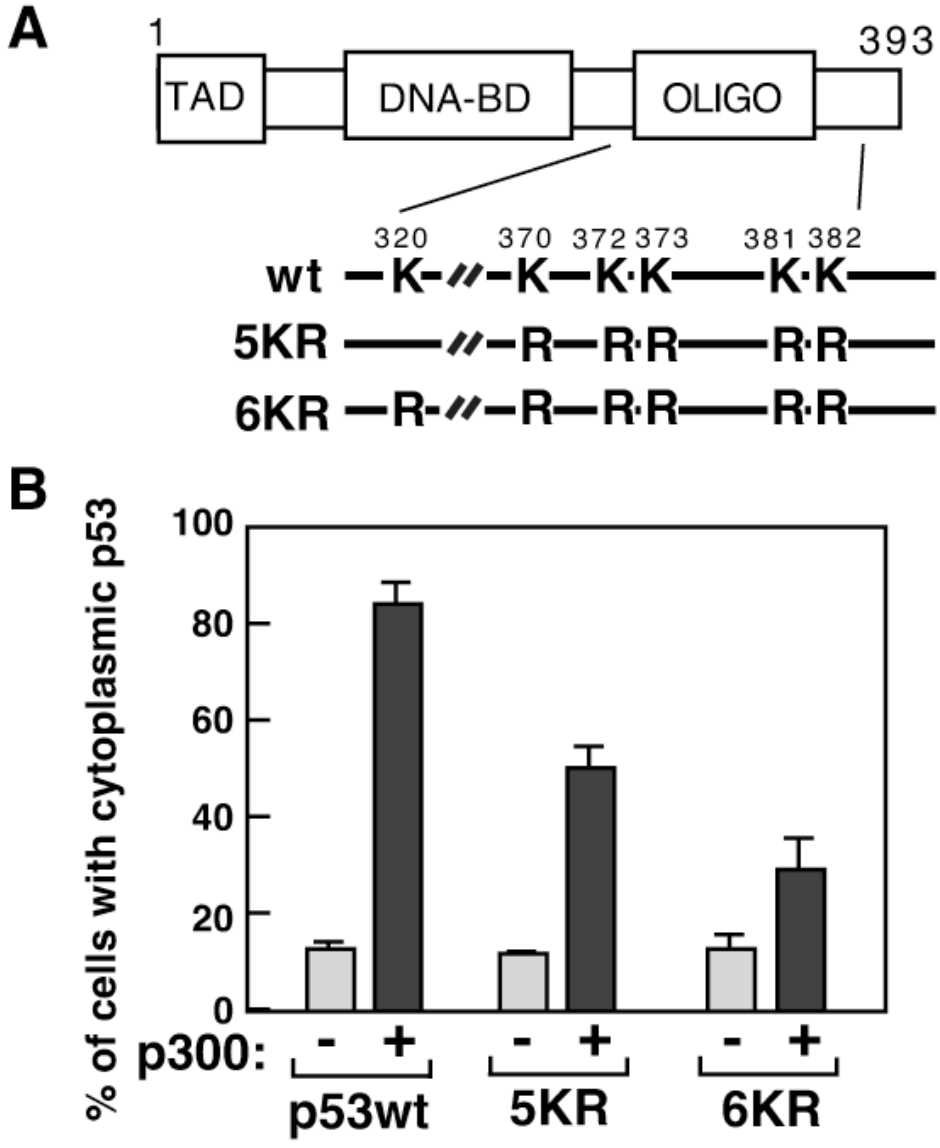
80
60
40
20
0
p53wt
p53wt
+p300
p53wt
+MDM2

Figure3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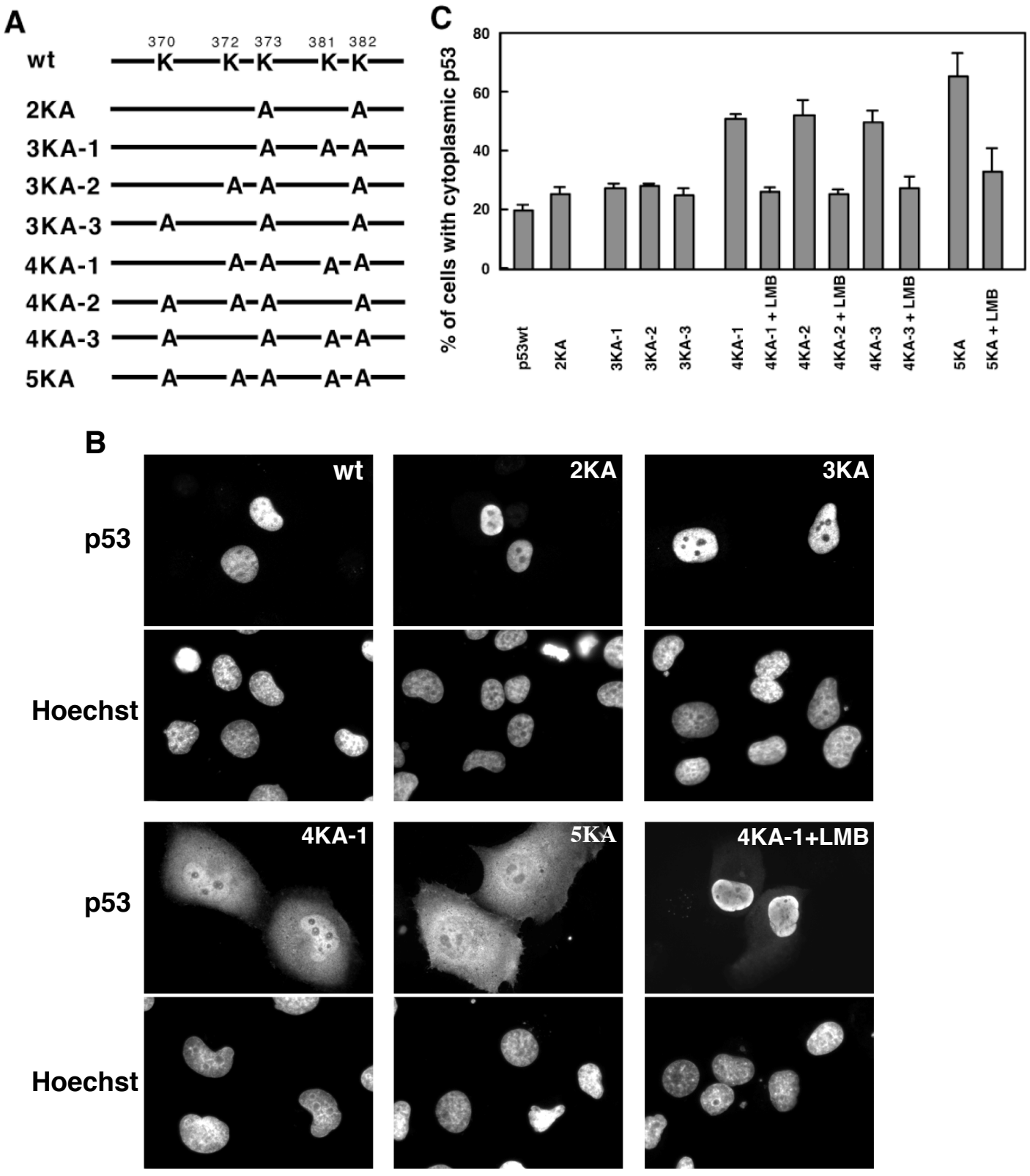
393
TAD
DNA-BD
OLIGO
320
370
372 373
381 382
wt
5KR
6KR
B
% of cells with cytoplasmic p53
100
80
60
40
20
0
p300:
-
+
p53wt
5KR
6KR

Figure4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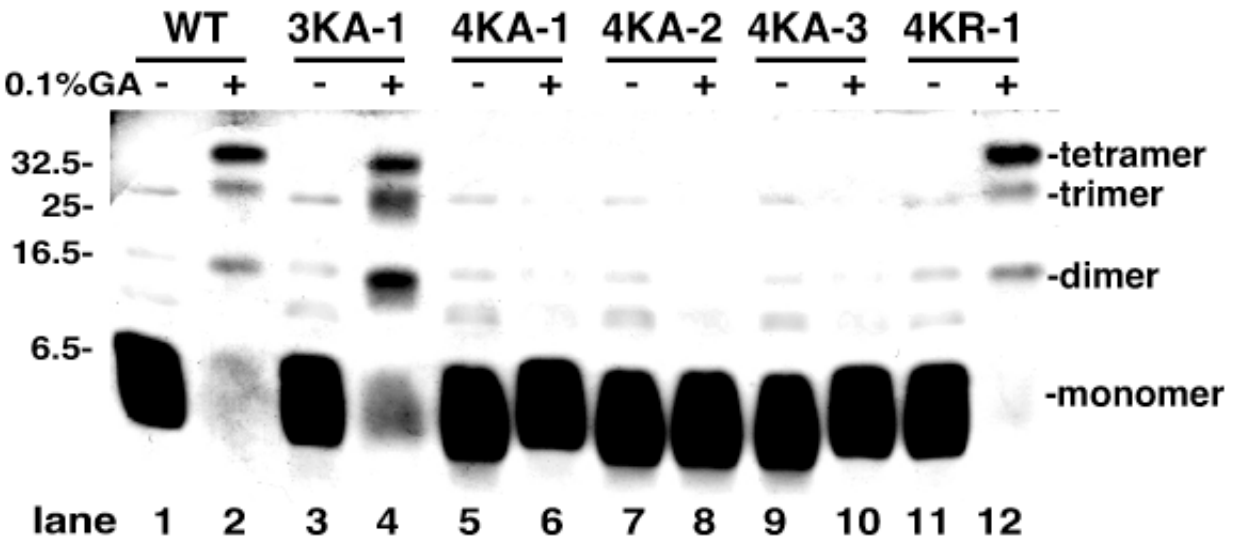
wt K K K K K
2KA A A
3KA-1 A A A
3KA-2 A A A
3KA-3 A A A
4KA-1 A A A A
4KA-2 A A A A
4KA-3 A A A A
5KA A A A A
C
% of cells with cytoplasmic p53
0
20
40
60
80
p53wt
2KA
3KA-1
3KA-2
3KA-3
4KA-1
4KA-1 + LMB
4KA-2
4KA-2 + LMB
4KA-3
4KA-3 + LMB
5KA
5KA + LMB
B
p53
Hoechst
wt
2KA
3KA
4KA-1
5KA
4KA-1+LMB

Figure5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4KA-1
4KA-2
4KA-3
4KR-1
0.1%GA - + - + - + - + - + - +
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25-
16.5-
6.5-
-tetramer
-trimer
-dimer
-monomer
lane 1 2 3 4 5 6 7 8 9 10 11 12